0022-538X/78/0025-0073$02.00/0
JOURNAL OF VIROLOGY, Jan. 1978, p. 73-77

Vol. 25, No. 1
Printed in U.S.A.

Mutator and Antimutator Phenotypes of Suppressed Amber Mutants in Genes *32*, *41*, *44*, *45*, and *62* in Bacteriophage T4

SUSAN M. WATANABE AND MYRON F. GOODMAN*

Department of Biological Sciences, University of Southern California, Los Angeles, California 90007

Received for publication 22 August 1977

Bacteriophage T4 genes *32*, *41*, *44*, *45*, *56*, and *62* are essential to DNA replication. Amber mutants (suppressed by su^+1, su^+2, or su^+3 bacteria) in these genes were examined for any mutator or antimutator effects on the reversion of a transition mutation. In every case except for mutations in gene *56*, elevated or lowered error frequencies were observed. These results indicate the importance of all of the replicative proteins in the determination of error frequency.

Recent studies by Morris et al. (22) have indicated that an in vitro assay containing bacteriophage T4 gene products *32*, *41*, *43*, *44*, *45*, and *62* is capable of synthesizing DNA and possesses many of the features expected in vivo (i.e., requirement for ribonucleoside triphosphates and ability to replicate extensively and processively from a single-stranded circular template). These gene products, as well as gene *56* products, were earlier demonstrated (15, 27) to be intimately involved in the replication process through mutations that revealed a phenotype termed DO, characterized by virtually no DNA synthesis under nonpermissive conditions. In the analysis of mutational mechanisms, these essential gene products, which appear to be the fundamental components of the replication machinery, may be particularly illuminating since perturbation of their function can logically be expected to perturb the replication machinery, which in turn may affect the fidelity of replication.

Indeed, earlier studies on some of these bacteriophage gene products have revealed a profound effect on the mutation rate in vivo. In particular, mutations in T4 gene *43* (DNA polymerase) have produced increased (25) and decreased (13) mutation frequencies of as much as two orders of magnitude. Lesser effects have been seen with mutants in genes *32* (DNA unwinding protein) (3, 4, 12, 19), *44* (ATPase) (3), and *42* (dCMP hydroxymethylase) (9, 12, 26). Mutations in genes *30* (ligase) and *46* and *47* (exonucleases), phenotypically characterized as DA (DNA arrest), have also yielded alterations in mutation frequency (4, 5, 18).

It was of interest, therefore, to examine the effect on fidelity of mutations in the heretofore untested genes *41*, *45*, and *62* that have been implicated by Alberts as fundamental elements in the replication process in vitro (2, 22). In addition, the studies here describe effects on fidelity due to mutations in the previously unexamined gene *56* (demonstrated to be essential in vivo), as well as effects due to mutations in genes *32* and *44*. Experiments described here were performed on amber and temperature-sensitive phage strains grown in three different hosts that were either su^+1 (serine), su^+2 (glutamine), or su^+3 (tyrosine). By using different suppressor bacteria, we could establish three different missense mutants per amber mutant simply by varying the amino acid inserted (see, for example, L. Reha-Krantz and M. J. Bessman, J. Mol. Biol., in press). Reversion of the *rIIB* marker *UV199* (14) was used to analyze the effects of these *am* and *ts* mutations on the transition adenine:thymine → guanine:cytosine (8).

MATERIALS AND METHODS

Media. Phage stocks were grown in M9a (10) and titered on tryptone agar (10 g of tryptone [Difco]-5 g of NaCl-13 g of agar [Difco] per liter of water), using a tryptone overlay (10 g of tryptone [Difco]-5 g of NaCl-8 g of agar [Difco] per liter of water).

Phage and bacteria. The T4 B *rII UV199* mutant was a gift from J. W. Drake. The seventeen *am* or *ts* mutants in the replicative genes *32*, *41*, *44*, *45*, *56*, and *62* were a gift from W. Wood's laboratory. Double mutants containing the *am* lesion and the *rII* lesion were constructed in this laboratory. Wild-type *Escherichia coli* B (CGSC no. 2) and CR63 (CGSC no. 3591) were obtained from the *E. coli* Genetic Stock Center at the Yale University School of Medicine. CR63(λ) was obtained from M. J. Bessman, and C600 and Ymel were from H. Shizuya.

Preparation of phage stocks. All stocks were grown from single plaques in M9a at 32°C in *E. coli* CR63. A total of 5 ml of *E. coli* CR63 organisms were grown to 2×10^8 cells per ml from a 1,000-fold dilution of an overnight culture. The infected bacteria were grown on rotary shakers for 6 h and then chloroformed to complete lysis.

Reversion analysis. A total of 3 ml of *E. coli*

CR63 (*su⁺1*), C600 (*su⁺2*), or Ymel (*su⁺3*) organisms were grown in M9a to 2×10^8 cells per ml from a 1,000-fold dilution of an overnight culture and then inoculated with stabs of single plaques from the CR63 stocks. The infected bacteria were grown on a rotary shaker at 32°C and then chloroformed after 6 h. At least three cultures were grown in parallel for any phage strain in each given host. At least three stocks of the control genotype, *am⁺-rUV199*, were also grown in parallel in each bacterial background.

To test for *rII* revertant frequencies, the stocks were plated at different dilutions on CR63 and CR63(λ). The frequency of amber revertants was always monitored by plating on *E. coli* B (*su⁻*); the amber reversion frequencies were no greater than 10^{-7}, except for *amN57* (gene *41*) and *amNG223* (gene *41*), where a reversion frequency of 10^{-4} was observed. To ensure a comparable efficiency of plating between different suppressors, phage were also plated on the appropriate bacterial hosts used to grow the phage stock being examined. In no case did these titers vary significantly from titers determined on CR63.

Statistical analysis. The *t* test was used to define at what probability level the reversion frequencies for the *rII-am* double mutants could be declared as different from the reversion frequency of the *rII-am⁺* single mutant. In every case in which the *t* test was used, the data for any mutant strain were treated en masse. This treatment should underestimate the probability of significant differences since, in fact, mean reversion frequencies tend to fluctuate in unison from day to day. Therefore, although an *rII-am* double mutant may consistently revert more frequently than an *rII-am⁺* single mutant in a single day's reversion analysis, all of the reversion frequencies of the single and double mutants taken en masse show more overlap.

RESULTS

The effects on the *rII* marker's reversion frequency due to the various amber and temperature-sensitive lesions are given in Table 1. Note that most of the amber mutants in the three different suppressor backgrounds have *r⁺* revertant frequencies approximately the same as that of *rII-am⁺*. Nevertheless, there are several amber mutants that endow their *rII* marker with significantly higher or lower reversion frequencies, depending on the host suppressor.

Two gene *32* amber mutants, *amA453* and *amNG461*, show significant mutator phenotypes in *su⁺2* and *su⁺3* bacterial hosts. A small, but statistically significant, antimutator effect is seen in the gene *32 amE315* mutant when suppressed by *su⁺1* and *su⁺2* bacteria. *amN57*, a mutant of gene *41*, confers a significantly higher

TABLE 1. *Effects of mutations in genes 32, 41, 44, 45, 56, and 62 on the reversion frequencies of rUV199*

Gene	Allele	*rUV199* reversion frequency[a] (*rII-am/rII-am⁺*)								
		su⁺1			*su⁺2*			*su⁺3*		
		Range	Avg[b]		Range	Avg[b]		Range	Avg[b]	
32	*amA453*	2.0–1.0	1.6	(3)	15–13	14.5[c]	(3)	11–4	7[c]	(3)
	amNG364[d]	1.4–0.1	0.7	(3)	1.8–0.8	1.1	(3)			
	amE315[d]	0.5–0.3	0.4[c]	(6)	0.6–0.4	0.5[c]	(3)			
	amNG461	2.0–1.7	1.9	(3)	40–4	14[c]	(7)	5–3	4[c]	(3)
	tsL67	1.3–0.3	0.7	(6)	1–0.6	0.9	(6)	1–0.6	0.8	(3)
41	*amN57*	24–8	13[c]	(10)	8–6	7	(3)	5–4	4.5[c]	(2)
	amN81	0.9–0.7	0.8	(3)	2–1.8	2	(3)	1.6–1.2	1.5	(3)
	amNG223	2.3–.09	0.6	(10)	1.5–0.7	0.8	(3)	5.3–1	3.0	(3)
62	*amE1140*	0.7–0.4	0.5	(3)	0.6–0.3	0.5	(3)	2.6–1.3	1.8	(3)
	amE1165x3	3.0–1.7	2.8	(3)	37–6	14[c]	(10)	3.4–3	3.3	(3)
	amNG424x3	1.8–0.4	1.3	(3)	10–2.7	4.7[c]	(11)	2.6–1.3	1.8	(3)
44	*amN82*	0.7–0.3	0.5	(3)	2.7–1	1.5	(3)	6.8–4	5[c]	(3)
	amE2057	1.0–0.3	0.6	(3)	11–5	8.7[c]	(11)	12–4	9[c]	(3)
	amNG485	0.6–0.5	0.5	(3)	11–5	9[c]	(3)	28–3	11[c]	(7)
45	*amNG18*	0.5–0.12	0.23[c]	(13)	3–1.4	2	(3)	2.4–1.6	2.1	(3)
56	*amE51*	0.8–0.4	0.7	(3)	1.9–0.3	0.4	(3)	1.4–0.9	1	(3)
	tsA90x5	2.0–0.6	0.9	(3)	1.8–0.5	0.8	(3)	1.2–0.6	0.9	(3)

[a] The *rUV199* frequency employed in the denominators is from the control stocks grown and titrated in parallel.

[b] Numbers in parentheses indicate the numbers of trials.

[c] Reversion frequency was found to be significantly different by the *t* test (see text and Table 2).

[d] Repeated attempts with different *su⁺3* bacteria failed to yield phage stocks with titers high enough to give significant numbers.

reversion frequency when grown in *su⁺1, and su⁺3* backgrounds. Gene *62* mutants *amE1165x3* and *amNG424x3* are fairly strong mutators in *su⁺2* background. Gene *44* mutant *amE2057* also elevates reversion frequencies in *su⁺2* and *su⁺3*, as do two other gene *44* mutants, *amN82* in *su⁺3* and *amNG485* in *su⁺2* and *su⁺3*. Finally, *amNG18*, a gene *45* mutant, significantly lowers the reversion frequency when suppressed by *su⁺1* bacteria. Levels of statistical significance and reversion frequencies are given in Table 2.

These effects are not due to any unrecognized lesion since reversion of the amber locus is accompanied by a return to control (i.e., *rII-am⁺*) reversion frequencies. This was established by growing, in the appropriate *su⁺* host, three amber revertant phage stocks per original amber strain with three *rUV199* phage stocks as controls. The frequency of *rII* to *r⁺* reversion was checked by titering on CR63 and CR63(λ); the absence of the amber locus was ascertained by checking the efficiency of plating on *E. coli* B (*su⁻*). No significant differences were seen between the amber revertant *rUV199* (data not shown) and the control *rUV199* (Table 2) reversion frequencies.

There is evidence to indicate that the *rIIB* protein may bind to DNA, making the gene less than ideal as a neutral marker (11). Although selection due to interaction between the *rIIB* gene product and the replicative proteins cannot be directly ruled out, it can nevertheless be declared improbable for several reasons. First, the results given here indicate that, in most cases, no significant differences in marker reversion frequencies exist between suppressed *rII-am* and *rII-am⁺*. A missense mutation (e.g., a suppressed amber lesion), therefore, does not automatically dictate by its presence differences in viability between *rII UV199* and its *r⁺* revertant. (For example, *tsL67* confers a reversion frequency that is equal to the *rII-ts⁺* in all of the suppressor hosts. This argues against the other gene *32* mutants' mutator or antimutator phenotypes being due to a generalized effect of the presence of a lesion.) Second, in most cases the increases or decreases in reversion frequencies in one suppressor background were not accompanied by a similar effect in another suppressor background. A missense mutation at a specific locus in a replication protein, therefore, does not consistently confer an increased viability for either the *rII UV199* or the *r⁺* revertant. Third, the phage stocks were grown in hosts that do not permit the expression of the *rII* phenotype, so *rII UV199* and its *r⁺* revertant are phenotypically indistinguishable.

DISCUSSION

Table 2 shows the important roles played by the replicative proteins *P32, P41, P44, P45,* and *P62* in determining the fidelity of replication. Although no significant effects were observed in the gene *56* mutants, the possibility still remains

TABLE 2. *rUV199 reversion frequencies and significance levels for mutators and antimutators*

Host suppressor	Mutant allele	Gene	Avg reversion frequency per 10^8 phage[a]	Standard deviation of the mean	Significance level[b]
su⁺1	*am⁺*		56 (19)	6	
	amE315	*32*	27 (6)	5	<0.01
	amN57	*41*	783 (10)	16	<0.0005
	amNG18	*45*	16 (13)	3	<0.0005
su⁺2	*am⁺*		70 (20)	11	
	amA453	*32*	432 (10)	138	<0.0005
	amE315	*32*	26 (3)	5	<0.10
	amNG461	*32*	650 (7)	312	<0.005
	amE1165x3	*62*	97 (3)	29	<0.20
	amNG424x3	*62*	680 (10)	135	<0.0005
	amE2057	*44*	185 (11)	53	<0.005
	amNG485	*44*	332 (11)	62	<0.0005
			543 (3)	132	<0.0005
su⁺3	*am⁺*		133 (15)	16	
	amA453	*32*	500 (3)	190	<0.0005
	amNG461	*32*	270 (3)	59	<0.005
	amN57	*41*	330 (2)	70	<0.0005
	amN82	*44*	390 (3)	72	<0.0005
	amE2057	*44*	630 (3)	150	<0.0005
	amNG485	*44*	1,060 (7)	178	<0.0005

[a] Numbers in parentheses indicate the numbers of trials.
[b] Significance levels were determined by the *t* test (see text).

that a larger sampling of gene *56* mutants may demonstrate mutator or antimutator phenotypes.

The elevated reversion frequencies observed for the two gene *32* mutants, *amA453* and *amNG461*, are not unexpected in view of recent reports (3, 4, 12, 19). The significantly lowered error frequency for the gene *32* mutant *amE315* is, however, somewhat anomalous. One may logically expect that the determination of fidelity by the gene *32* protein stems from its ability to bind to single-stranded DNA, thereby providing a suitable template (1, 6, 7), and/or to interact with the DNA polymerase (17, 21, 24). It has been observed previously that mutator, but not antimutator, phenotypes result from disturbing the function of the gene *32* protein (3, 4, 12, 19). However, there has been some evidence to show that a decreased stabilization of the helix immediately preceding the incoming nucleotide may lower the probability of an error in replication (Bessman and Reha-Krantz, J. Mol. Biol., in press). Such a change could occur through the creation of a gene *32* protein with a higher affinity for single-stranded DNA and, in fact, proteolytic cleavage of the gene *32* protein in vitro has been observed to lower the DNA melting temperature (20).

The activities of the other replicative proteins from genes *41, 44, 45,* and *62* are, unfortunately, not as clearly understood as are those of gene *32* protein. Nevertheless, the findings here may serve as clues to their function. For example, it has been suggested that the *44–62* complex with its ATPase activity may serve as part of a preincorporation "kinetic-proofreading" system (16), providing for an increase in the fidelity of base pairing before polymerization. A disturbance in the *44–62* complex through a mutation may result in a mutator, rather than an antimutator, phenotype. We have, in fact, found only mutators thus far. *P45* has been implicated in increasing the processivity of *P43* (DNA polymerase) (22). A mutation may perturb this protein, causing it to lose some of its activity (i.e., decrease processivity of *P43*). A decrease in polymerization or processivity may be concomitant with an increase in exonuclease activity; both have been linked to decreased error frequency (23). Perhaps it is by this mechanism that the antimutagenic effects in the gene *45* mutant *amNG18* occur.

The above models are, of course, only conjectures. However, the results of this study clearly indicate the importance of all of the replicative proteins in the determination of the fidelity of DNA replication in vivo. Since evidence points toward the six replicative proteins working synergistically (2, 22), a mutation in any one of the proteins could easily interfere with the activities of the rest of the complex. The possibility of this interference leading to a change in the error frequency should be appreciable; this has proved to be true among the mutants studied here.

However, one should note that the effects seen here and in earlier work (3–5, 9, 12, 18, 19) have not been as dramatic as those seen in the gene *43* protein (DNA polymerase) (13, 25). This may indicate that the DNA polymerase is the most crucial element in the replication complex in determining fidelity. Nevertheless, the importance of all of the protein products involved in DNA synthesis cannot be overemphasized since gene products *32, 41, 43, 44, 45,* and *62* function as an interacting complex in replication, perhaps making error frequencies not a result of a disturbance in any one gene product's function as much as a disturbance of the function of the entire complex.

ACKNOWLEDGMENTS

We thank Randi Hopkins for invaluable advice and unflagging encouragement.

This work was suported by Public Health Service grants GM21422 and CA17358 from the National Institute of General Medical Sciences and the National Cancer Institute, respectively.

LITERATURE CITED

1. **Alberts, B. M., and L. Frey.** 1970. T4 bacteriophage gene 32: a structural protein in the replication and recombination of DNA. Nature (London) **227**:1313–1318.
2. **Alberts, B. M., C. F. Morris, D. Mace, N. Sinha, M. Bittner, and L. Moran.** 1975. Reconstruction of the T4 bacteriophage DNA replication apparatus from purified components. Systems for replication of DNA *in vitro*, p. 241–269. *In* M. Goulian, P. Hanawalt, and C. F. Fox (ed.), ICN-UCLA Symposium on Molecular and Cellular Biology. W. A. Benjamin, Inc., Reading, Mass.
3. **Bernstein, C., H. Bernstein, S. Mutti, and B. Strom.** 1972. Stimulation of mutation in phage T4 by lesions in gene *32* and by thymidine imbalance. Mutat. Res. **16**:113–119.
4. **Bernstein, H.** 1971. Reversion of frameshift mutations stimulated by lesions in early function genes of bacteriophage T4. J. Virol. **7**:460–466.
5. **Campbell, D. A., and L. Rowe.** 1972. Enhanced recovery of *rII* mutations in the absence of selection in a ligase-negative mutant of bacteriophage T4. Mutat. Res. **15**:361–369.
6. **Carroll, R. B., K. E. Neet, and D. A. Goldthwait.** 1972. Self-association of gene *32* protein of bacteriophage T4. Proc. Natl. Acad. Sci. U.S.A. **69**:2741–2744.
7. **Carroll, R. B., K. E. Neet, and D. A. Goldthwait.** 1975. Studies on the self-association of bacteriophage T4 gene *32* protein by equilibrium sedimentation. J. Mol. Biol. **91**:275–291.
8. **Champe, S., and S. Benzer.** 1962. Reversal of mutant phenotype by 5-fluorouracil: an approach to nucleotide sequence in messenger RNA. Proc. Natl. Acad. Sci. U.S.A. **48**:532–546.
9. **Chiu, C., and R. Greenberg.** 1973. Mutagenic effect of temperature-sensitive mutants of gene 42 (dCMP hy-

droxymethylase) of bacteriophage T4. J. Virol. **12**:199–220.

10. **Clowes, R. C., and W. Hayes.** 1968. Experiments in microbial genetics, p. 187–188. Blackwell Scientific Publications, Oxford.
11. **Colin, M., N. Sinha, and B. M. Alberts.** 1977. Intracellular DNA protein complexes from bacteriophage T4-infected cells isolated by a rapid two-step procedure. J. Biol. Chem. **252**:2734–2741.
12. **Drake, J. W.** 1974. The genetic control of spontaneous and induced mutation rates in bacteriophage T4. Genetics **73**(Suppl.):45–64.
13. **Drake, J. W., and E. F. Allen.** 1968. Antimutagenic DNA polymerases of bacteriophage T4. Cold Spring Harbor Symp. Quant. Biol. **33**:339–344.
14. **Drake, J. W., E. F. Allen, S. Forsberg, R. Preparata, and E. Greenberg.** 1969. Genetic control of mutation rates in bacteriophage T4. Nature (London) **221**:1128–1132.
15. **Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevally, R. Edgar, M. Sussman, G. Denhardt, and A. Leilansis.** 1963. Physiological studies of conditional lethal mutants of bacteriophage T4D. Cold Spring Harbor Symp. Quant. Biol. **28**:375–392.
16. **Hopfield, J. J.** 1974. Kinetic proofreading: a new mechanism for reducing errors in biosynthetic processes requiring high specificity. Proc. Natl. Acad. Sci. U.S.A. **71**:4135–4139.
17. **Huberman, J. A., A. Kornberg, and B. M. Alberts.** 1971. Stimulation of T4 bacteriophage DNA polymerase by the protein product of T4 gene *32*. J. Mol. Biol. **62**:39–52.
18. **Koch, R. E., and J. W. Drake.** 1973. Ligase-defective bacteriophage T4. I. Effects on mutation rates. J. Virol. **11**:35–40.
19. **Koch, R. E., M. K. McGraw, and J. W. Drake.** 1976. Mutator mutations in bacteriophage T4 gene *32* (DNA unwinding protein). J. Virol. **19**:490–494.
20. **Moise, H., and J. Hosoda.** 1976. T4 gene *32* protein model for control of activity at the replication fork. Nature (London) **259**:455–458.
21. **Molineux, I. J., and M. L. Gefter.** 1974. Properties of *Escherichia coli* DNA binding (unwinding) protein: interaction with DNA polymerase and DNA. Proc. Natl. Acad. Sci. U.S.A. **71**:3858–3862.
22. **Morris, C. F., N. K. Sinha, and B. M. Alberts.** 1975. Reconstruction of bacteriophage T4 DNA replication apparatus from purified components: rolling circle replication following *de novo* chain initiation on a single-stranded circular DNA template. Proc. Natl. Acad. Sci. U.S.A. **72**:4800–4804.
23. **Muzyczka, N., R. L. Poland, and M. J. Bessman.** 1972. Studies on the biochemical basis of spontaneous mutation. J. Biol. Chem. **247**:7116–7122.
24. **Sigal, N., H. Delius, T. Kornberg, M. L. Gefter, and B. Alberts.** 1972. A DNA-unwinding protein isolated from *E. coli*: its interaction with DNA and with DNA polymerases. Proc. Natl. Acad. Sci. U.S.A. **69**:3537–3541.
25. **Speyer, J. F.** 1965. Mutagenic DNA polymerase. Biochem. Biophys. Res. Commun. **21**:6–8.
26. **Williams, W. E., and J. W. Drake.** 1977. Mutator mutations in bacteriophage T4 gene *42* (dHMC hydroxymethylase). Genetics **86**:501–511.
27. **Wood, W.** 1974. *In* R. C. King (ed.), Handbook of genetics, vol. 1, p. 327–331. Plenum Press, New York.